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ORIGINAL PAPER

Overexpression of squalene synthase in *Withania somnifera* leads to enhanced withanolide biosynthesis

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Abstract Genetic engineering of secondary metabolic pathways is an emerging area of research for production and improvement of natural products in plant biotechnology. Here, we describe a systematic approach to manipulate a key regulatory step of isoprenoid biosynthetic pathway in *Withania somnifera* to study its effect on withanolide production. We generated T_0 *W. somnifera* plants overexpressing squalene synthase (*WsSQS*) by *Agrobacterium tumefaciens* mediated transformation, which were analyzed by Gus biochemical assay and PCR of hygromycin phosphotransferase (*hpt*II) and *WsSQS*. qRT-PCR analyses of various transformed tissues indicated 2–5 fold increase in *WsSQS* transcripts in both T_0 and T_1 generations. The tissue specific protein expression studies revealed 2–3 fold increase in WsSQS, which was further confirmed by enzyme activity. These observations were corroborated with the 1.5–2 fold increase in total withanolide content of the transformed tissues. However, in leaf tissue, the levels of Withaferin A and Withanolide A increased significantly up to 4–4.5 fold. These findings demonstrate genetic engineering of isoprenoid pathway in *W. somnifera* resulting in enhanced production of withanolides, and also provide insights into such metabolic pathways for their manipulation to improve the pharmacological content of different medicinally important plants.

Keywords *Agrobacterium tumefaciens* · Solanaceae · Squalene synthase · *Withania somnifera* · Withanolides

Introduction

Isoprenoids are one of the largest groups of natural products with structurally diverse carbon skeleton present in all forms of life. They include several biologically active and commercially important compounds like sterols, steroidal sapogenins, alkaloids and lactones. Withanolides are one such class of naturally occurring steroidal lactones, commonly found in members of Solanaceae. A majority of these secondary metabolites (SMs) are concentrated in *Withania somnifera*, characterized by C_{28} ergostane backbone with a side chain of C_9 having six-membered lactone ring which is a distinctive feature of these metabolites responsible for most of their biological activities.

W. somnifera (L.) Dunal, commonly known as Indian ginseng, is principally recognized for its medicinal value in Ayurveda and has been well investigated for its antiserotogenic, adaptogenic and anticancer activity (Winters 2006). These pharmacological efficacies of the plant are attributed to different withanolides which are actively involved in various plant processes such as defense, signaling, flavor and fragrance, and also accounts for hormonal, antibiotic, insecticidal and therapeutic activities of the plant. Withanolides are widely known for their antioxidant, anti-inflammatory, immunomodulating, antistress and rejuvenating effects (Bhattacharya et al. 2001; Gupta et al. 2003).

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For large scale cultivation, in vitro regeneration has been widely studied in *W. somnifera* using various explants like axillary meristems (Roja et al. 1991), mature leaf (Joshi and Padhya 2010), shoot tips (Ray and Jha 2001), hypocotyl (Rani et al. 2003), and others (Ghimire et al. 2010; Logesh et al. 2010; Sharma et al. 2010). But there are few reports where nodal explants have been used for the regeneration and shoot multiplication in *W. somnifera* (Kumar et al. 2011; Sivanandhan et al. 2011). *Agrobacterium tumefaciens* mediated genetic transformation of *W. somnifera* using nodal explants have been shown to produce more number of transgenic shoots directly from nodal explants within a short period of time (Udayakumar et al. 2014). Apical and nodal explants have resulted in higher transformation efficiency in *W. somnifera* with proper concentration of phytohormones (Patel et al. 2014). In a present study, apical and nodal segments were used as explants for transformation.

W. somnifera is a slow growing shrub producing minute quantity of withanolides which depends on the physiological and developmental stage of the plant (Oksman-Caldentey and Inze 2004). Such a low abundance is incapable of meeting the present economic demand for medicinal formulations. In this regard, chemical synthesis of these plant natural products were attempted, however, sheer structural complexity and the specific stereochemical requirements of the compounds resulted in their low yield (Jana et al. 2011). Plant cell cultures had been set up as an alternative for extraction of SMs which ended up in limited commercial success and technological issues such as maintenance of large bioreactors and cultivation conditions (Oksman-Caldentey and Inze 2004).

This limitation, however, may be overcome by looking into the biosynthetic pathway of withanolides (Fig. 1) and using genetic engineering as a tool to manipulate the crucial steps of this metabolic network to increase their yield. The first committed step which diverts the carbon flux away from the central isoprenoid pathway and towards withanolide biosynthesis is the formation of squalene; catalyzed by a 47 kDa membrane associated enzyme, squalene synthase (SQS; EC 2.5.1.21) (Gupta et al. 2012). The substrate for this enzyme, farnesyl diphosphate (FPP), originates from isoprenoid biosynthetic pathway and can be channeled towards squalene accumulation which is supposed to be the first precursor of triterpenoids. SQS catalyses head to head condensation of two FPP molecules to form presqualene diphosphate which undergo rearrangements and cyclopropyl ring opening to form squalene in the presence of NADPH and Mg^{2+} (Pandit et al. 2000). Squalene oxidizes in presence of NADPH to afford squalene 2, 3-epoxide, subsequently cyclizing into lanosterol which acts as a backbone structure for various steroidal triterpenoids (Mirjalili et al. 2009). The complete biosynthetic pathway of withanolides is still unclear but they are supposed to be derived from 24-methylenecholesterol (Lockley et al. 1976).

Since SQS acts at a regulatory branch point, it has attracted considerable interest as a possible genetic engineering target for diverting the metabolic flux to promote SM biosynthesis in plants. Many approaches have been investigated to understand the regulatory role of SQS in sterol biosynthesis using SQS mutants (Tozawa et al. 1999), fungal elicitors (Vogeli and Chappell 1988) and specific inhibitors of SQS (Wentzinger et al. 2002). Genetically improved *Withania* with increased levels of withanolides would have a profound impact on pharmaceutical industries. We show here that upregulation of squalene synthase in triterpenoid pathway leads to generation of transformed *Withania* plants by *Agrobacterium* mediated transformation, having normal phenotype and enhanced withanolides content as compared to the controls. These modified lines and the approach used are valuable for developing improved cultivars of beneficial crops.

Materials and methods

Plant material and propagation

The seeds of *W. somnifera* (L.) Dunal were procured from Vindhya herbals, Bhopal, MP, India. Seeds were surface sterilized, germinated, and micropropagation was achieved according to the protocol described already (Patel et al. 2014). In brief, the sterilized seeds were inoculated on half-strength solidified MS (Murashige and Skoog) medium (Murashige and Skoog 1962) and incubated in dark for 15 days. The germinated seed were transferred to half-strength liquid MS medium for further development. The grown seedlings were multiplied in proliferation medium (MS supplemented with 0.1 mg l^{-1} kinetin and 0.2 mg l^{-1} 6-BAP) and rooted in rooting medium (half strength MS liquid medium containing 2 mg l^{-1} IBA). The apical and nodal segments of 1 cm length containing a single node were excised from in vitro grown shoots and propagated separately to observe their response in the medium. Both the tissues resulted in shoot multiplication and regeneration, and there was no significant difference noticed with respect to the time of regeneration in the medium, number of multiple shoot formation, rooting time and morphology of root (data not shown). Therefore, both apical and nodal segments of 1 cm length were used as explants for transformation irrespective of their type.

Vector construction

Previously cloned full length squalene synthase from *W. somnifera* (*WsSQS*; GenBank GU732820) with an open

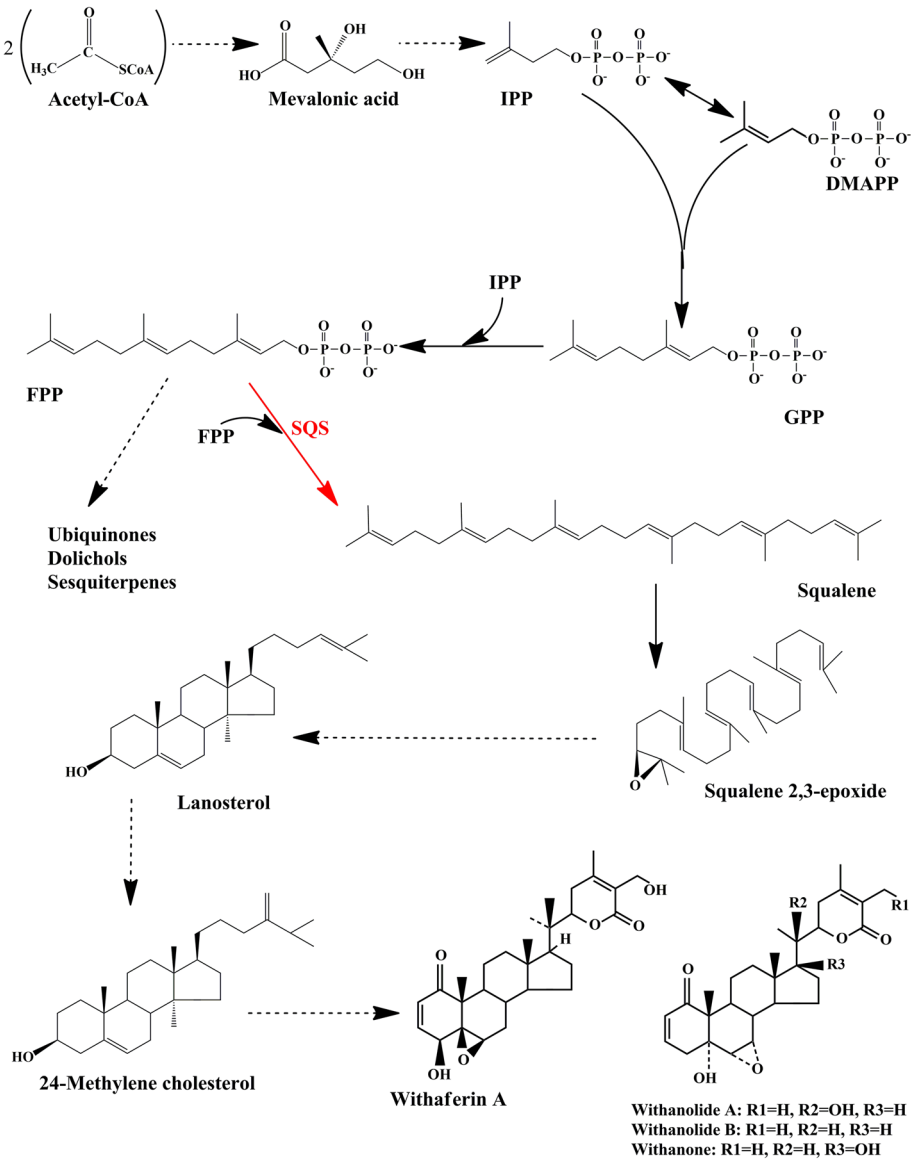

Fig. 1 Simplified scheme of withanolide biosynthetic pathway. Abbreviations: *IPP* Isopentenyl-5-pyrophosphate, *DMAPP* Dimethylallyl pyrophosphate, *GPP* Geranyl pyrophosphate, *FPP* Farnesyl pyrophosphate, and *SQS* Squalene synthase. *Dashed arrows* show that multiple steps are involved in the reaction

reading frame of 1242 bp (Gupta et al. 2012) was amplified using primers having sites for the restriction enzymes *Kpn*I and *Sac*I. The resulting fragment was positioned between cauliflower mosaic virus (CaMV) 35S promoter and a nopaline synthase (Nos) terminator in modified T-DNA region of pCAMBIA 1301 vector to ensure high level of gene expression (Patel et al. 2014). This vector contains a reporter gene namely *gus* (β–Glucuronidase) and a selectable marker *hpt*II (hygromycin phosphotransferase) gene imparting resistance to hygromycin B under the control of the constitutive CaMV 35S promoter to confirm transgene expression The construct was transformed into *A. tumefaciens* GV2260 for subsequent genetic transformation. *W. somnifera* explants transformed with *Agrobacterium* harboring the empty vector were used as control.

Genetic transformation of *W. somnifera*

Genetic transformation in *W. somnifera* was achieved by *A. tumefaciens* GV2260 carrying *WsSQS* in three sets of 30 explants each, according to the already described protocol (Patel et al. 2014). Essentially, the preconditioned explants were wounded and immersed in a freshly prepared bacterial suspension for 15 min, blotted dry to remove excess of bacterial culture and transferred to the proliferation medium. Cocultivation was carried out for 48 h in dark at 26 ± 2 °C. After 48 h, the cocultivated explants were washed with sterile distilled water containing 250 mg l^{-1} cefotaxime, blotted dry and shifted to proliferation medium supplemented with 250 mg l^{-1} cefotaxime. After 10 days, the explants were transferred to the selection medium

(proliferation medium containing 10 mg l^{-1} hygromycin B) and shoot development was monitored. Expression of the inserted genes in the transformed tissues was regularly observed by visualizing the β–Glucuronidase activity in different sectors of treated explants and growing shoots under a stereomicroscope (Leica MZ 125, Switzerland). The hygromycin B-resistant Gus-positive shoots were continuously maintained on selection pressure while the concentration of cefotaxime was reduced to 100 mg l^{-1} and then completely eliminated. To produce independent transformed lines, the shoots were detached from the transformed explant, cultured on proliferation medium and rooted onto the rooting medium.

Molecular identification of transformants

PCR (C1000 BIO-RAD thermal cycler, BIO-RAD, USA) was carried out using primers specific to the *hpt*II and *WsSQS*. Total genomic DNA was extracted from tissues of wild-type and hygromycin B-resistant transformed shoots by using plant DNA extraction kit (Hipura Plant Genomic Purification kit, Himedia, India). The *hpt*II gene specific forward and reverse primer sequences used were 5′-TCCTGCAAGCTCCGGATGCCTC-3′ and 5′-CGTGCACAGGGTGTCACGTTGC-3′, respectively. For *WsSQS* gene specific PCR, the forward primer was designed from the sequence of CaMV 35S promoter (GeneBank GQ336528.1; 5′-ACAGTCTCAGAAGACCAAAGGGCA-3′) to avoid endogenous *SQS* amplification, and reverse primer was designed from the 3′ terminal sequence of *WsSQS* (5′-GAGCTCCTAAGATCGGTTGCCAG-3′). The components of PCR reaction mixture were: 15 ng template DNA, 150 μM dNTPs, *hpt*II/*WsSQS* gene specific forward and reverse primers (0.66 pmol each), 0.5 U of *Taq* DNA polymerase in a total volume of 15 μl with 1X reaction buffer. The PCR reaction was carried out as follows: an initial denaturation at 94 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 1 min (for *hpt*II) and 1.6 min (for *WsSQS*) and a final 5 min extension at 72 °C. The amplified products were subjected to 1 % (w/v) agarose gel electrophoresis and visualized by ethidium bromide staining under UV.

qRT-PCR analysis

Total RNA was isolated from different tissues of *WsSQS* transformed lines and controls using Plant RNA Isolation Kit (Invitrogen, USA) as per manual instructions and treated with DNase using DNase I Digest kit (Sigma, USA). Total RNA (2 μg) was reverse transcribed into cDNA using AMV reverse transcription system (Promega, USA) with oligo dT primers in a 20 μL reaction volume. The reaction mixture was incubated for 1 h at 42 °C. For normalization of the relative expression data, ubiquitin gene (*UBC*) was employed as an internal standard using primer mix from Eurogentec (Belgium). To quantify the *WsSQS* transcripts, first-strand DNA was PCR amplified using gene-specific primers: SQS-F (5′-TTTATGATCGTGAATGGCACTTTTC-3′) and SQS-R (5′-AGCGGTTGAAACATGATGGAAC-3′) synthesized from Ws*SQS*. All qRT-PCR reactions were performed with SYBR Green Brilliant® II QPCR Master Mix (2× with low ROX, Stratagene, USA) on Mx 3000P instrument (Stratagene, USA) according to the manufacturer's instructions. PCR cycling conditions included a DNA denaturing stage of 95 °C for 10 min, followed by 40 cycles of 95 °C for 30 s, 55 °C for 45 s and 72 °C for 30 s. The amplified products were analyzed with MxPro software provided with the machine. Data were analyzed by comparative Ct method (Pfaffl 2001).

Indirect ELISA and western blot

Fresh tissues (500 mg each) of *WsSQS* transformed and control plants were ground and resuspended in 1 ml of phosphate buffered saline (PBS; 136 mM NaCl, 2 mM KCl, 8 mM Na_2HPO_4, 1 mM KH_2PO_4, pH 7.5) containing CHAPS (5 mM) to solubilize membrane proteins. Total protein quantity was estimated in the supernatant by Bradford assay using bovine serum albumin as standard. The polyclonal antibodies against purified WsSQS were raised in rabbits (New Zealand White) at National Toxicology Centre, Pune, India, and the antibody titer of the anti-SQS serum was determined. For detection of WsSQS in total plant protein, equal concentration of extracted protein (100 μL/well) was coated on 96 well polystyrene microtiter plate (Costar, USA) overnight at 4 °C followed by washings with PBST (PBS + 0.05 % Tween 20). Non-specific sites were blocked with blocking buffer (PBS + 1 % BSA) and incubated for 2 h at 37 °C. After washing thrice with PBST, primary antibody (rabbit polyclonal IgG against WsSQS) was added and incubated for 2 h at 37 °C. The unbound primary antibody was washed thrice with PBST and the plate was exposed to secondary antibody (goat anti-rabbit IgG-alkaline phosphatase conjugate, 1:20000) followed by incubation for 1–2 h at 37 °C. The presence of antigen was determined by the addition of enzyme specific substrate pNPP (*p*-Nitro Phenyl Phosphate; 1 mg ml^{-1}) and incubating for 45 min in dark for the color development. The reaction was stopped by adding 10 mM EDTA and the absorbance was measured at 405 nm using an xMark ELISA plate reader (BIO-RAD, USA). Detection limit of ELISA was determined by plotting a standard curve using the purified recombinant truncated WsSQS protein. The concentration of the WsSQS

present in total soluble protein extracted from control and *WsSQS* transformed plants were analyzed in each case of three replications from the standard curve.

For western blot, total crude protein (50 µg) from *WsSQS* transformed and control tissues was electrophoresed on 10 % SDS-PAGE and electro-transferred on to PVDF membrane using iBlot gel transfer system (Invitrogen) as per manufacturer's instructions, with recombinant truncated WsSQS used as a size marker. WesternBreeze kit (Invitrogen, USA) was used for further processing of the blot. Blot was placed in blocking solution and incubated at room temperature for 30 min on rotatary shaker. The membrane was rinsed and incubated with primary antibody solution for 1 h. The membrane was washed thrice and incubated in secondary antibody for 30 min. Signals were detected with ready to use 5-bromo-4-chloro-3-indolyl phosphate and nitrobluetetrazolium (BCIP/NBT) solution (Calbiochem, Germany).

WsSQS enzyme acitivity

The microsomal protein fractions were prepared from different tissues as already described (Vogeli and Chappell 1988). Essentially, 1 g of frozen tissue was homogenized in 10 mL of protein extraction buffer, filtered and centrifuged at 10,000 *g* for 25 min at 4 °C. The supernatant was again centrifuged at 100,000 g for 60 min to obtain the microsomal pellet. The pellet was resuspended in 200 µL of 100 mM Tris·Cl (pH 8), 1.5 mM DTT and 20 % glycerol, and the protein concentration were determined by Bradford method.

Assay for WsSQS enzyme was carried out with 10 µg microsomal protein according to the method described previously (Gupta et al. 2012). The enzyme activity was determined fluorimetrically by measuring NADPH depletion during the reaction on an LS 55 spectrofluorimeter (Perkin Elmer). The assay mixture was excited at 340 nm and emission was recorded in the range 400–500 nm with characteristic maxima around 460 nm corresponding to NADPH fluorescence. Excitation and emission slits were kept at 7.5 and 2.5 nm, respectively, with a scan speed of 100 nm min^{-1}. The reaction was carried out at 30 °C for 1 h and averaged fluorescence of 5 accumulated scans were recorded at regular time intervals. A standard curve was prepared by plotting fluorescence of commercially available NADPH (dissolved in 50 mM Tris–Cl; pH 8) at 460 nm against concentration.

In order to validate the enzyme reactions, squalene formed in each reaction was checked on GC–MS. Replicates of the above mentioned reactions, after 2 h of incubation, were extracted using tert-butyl methyl ether, and concentrated to 100 µL by bubbling dry nitrogen. The concentrate (1 µL) was injected on GC–MS (Agilent 5975C mass selective detector interfaced with an Agilent 7890A gas chromatograph) fitted with a capillary column HP-5 (25 m × 0.25 mm, film thickness 0.33 µm 5 % methylpolysiloxane cross-linked capillary column, Hewlett-Packard, USA) with a split ratio of 10:1. The injector temperature was set at 290 °C with helium as the carrier gas (10 ml min^{-1}). The oven temperature was programmed from 150 to 250 °C at 10 °C min^{-1} and from 250 to 310 °C at the rate of 5 °C min^{-1}, and maintained at final temperature for 5 min. The chromatogram obtained was compared with the authentic squalene (Sigma, USA) for its retention time and mass fragmentation pattern (see Supplementary Fig. 1). The squalene content was calculated from the standard curve plotted from the peak area versus concentrations of authentic squalene, and expressed as nmol mg^{-1} protein.

Withanolides extraction and LC–MS analysis

Dried tissues (100 mg each) were separately crushed to fine powder and percolated thrice with 5 ml methanol for 1 h under shaking conditions at room temperature. The extracts were pooled, filtered, concentrated under reduced pressure at 45 °C and thoroughly washed with double volume of n-hexane. The methanolic fraction was dried completely and further partitioned twice with water:chloroform (1:1). The chloroform fractions were pooled, concentrated and finally dissolved in 150 µl methanol. The samples were filtered and subjected to liquid chromatography. All the solvents used in the study were HPLC grade purchased from Fischer Scientific, USA.

LC–MS was performed on Waters Acquity UPLC system (Milford, MA, USA) with an Acquity UPLC® BEH C18 column (2.1 × 100 mm, 1.7 µm) attached to a positive ion elecrospray ionization-mass spectrometer (Waters) for the identification and quantification of withanolides in *W. somnifera* extracts. Separations were achieved using a binary gradient elution of water (solvent A) and acetonitrile (solvent B) with the following program carried out at 25 °C: 10 % B for 2 min; 45 % B for 8 min; 75 % B for 10 min; and 95 % B for 5 min, at a flow-rate of 0.4 ml min^{-1}, with a total run time of 25 min. External standards of different withanolides (Chromadex, USA) were used to construct calibrated graph of peak area versus withanolide concentration, being linear over 10 measurements at different concentrations.

Statistical analysis

All the experiments were repeated thrice and the data of each experiment were obtained in three replicates. Data were analyzed using Graphpad Prism 5 software (San Diego, California). Values are represented as mean ± SE.

Statistical analysis was performed using Student's *t* test to evaluate difference between the control and transformed plants. *P* values <0.05 are considered significant.

Results and discussion

Genetic transformation of *W. somnifera*

The apical and nodal explants derived from tissue culture raised *W. somnifera* were transformed with *WsSQS* with the aim of improving withanolide accumulation in the plant. Gus assay enabled the monitoring of expression of the introduced genes in planta, thus identifying the Gus-positive shoots (Fig. 2e–g). This was the first level of screening where the chimeric and untransformed shoots were identified and separated, while the Gus-positive transformants were allowed to flourish in presence of hygromycin B. Shoot elongation and multiplication were observed in some explants while many shoots turned necrotic indicating elimination of the untransformed tissues in the growth process. Green and living shoots were separated and cultured in fresh selection medium (Fig. 2b). The hormonal combination of 0.1 mg l^{-1} Kinetin and 0.2 mg l^{-1} 6-BAP supported quick and maximum shoot proliferation and multiplication of the transformed explants (Patel et al. 2014). Elimination of any remaining chimeric shoots having untransformed branches was carried out by performing Gus assay regularly with different sectors and tissues of the growing shoots. Green shoots were subsequently transferred onto the rooting medium (Fig. 2c). In this process, total 10 hygromycin B resistant transformed lines were recovered. The rooted T_0 transformed plants were successfully hardened and shifted to green house (Fig. 2d). These plants were normal in growth and appearance with no phenotypic aberrations.

The genomic DNA isolated from wild-type and hygromycin B-resistant T_0 plants were analyzed by PCR for the presence of *hpt*II and *WsSQS* genes which showed an amplification of ~600 bp (Fig. 3a) and ~1.6 kb (Fig. 3b) respectively. Out of 10 explants that survived on selection medium, only 6 transformed lines tested positive for PCR analysis, thus resulting in an overall transformation frequency of 6.66 %. The empty vector control and *WsSQS* transformed T_0 plants of same age, size and similar growth characteristics were taken into consideration for further

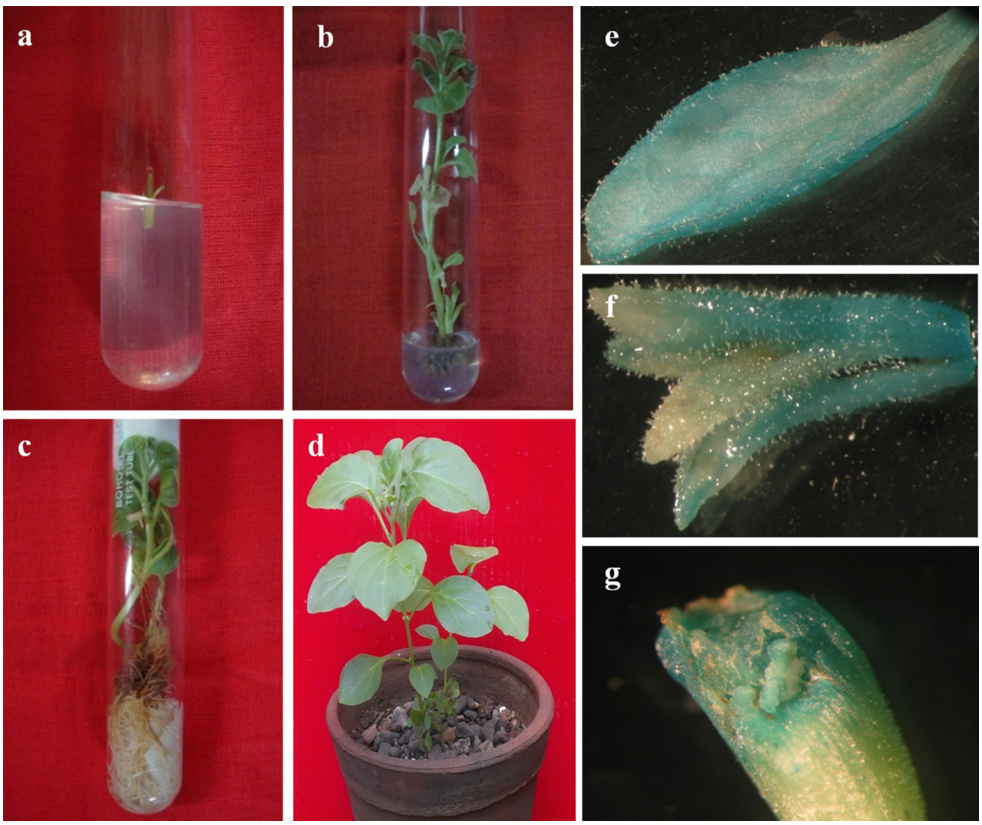

Fig. 2 Stages of genetic transformation of *W. somnifera*. **a** Nodal explant in proliferation media after 2 days of transformation; **b** shoot elongation and proliferation; **c** rooted plantlet; **d** successfully hardened transformed plant in green house; **e** Gus stained leaf tissue of T_0 plant; **f** Gus stained apical bud of T_0 plant; and **g** regenerating shoot of T_0 plantlet showing origin of Gus positive tissue

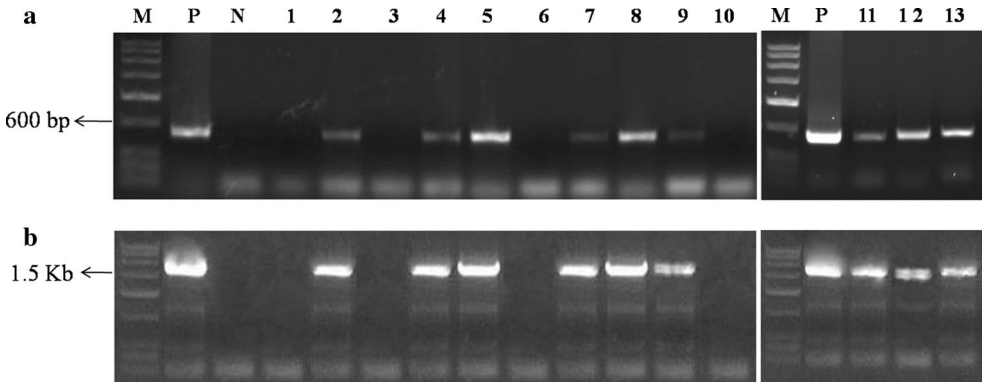

Fig. 3 Molecular analyses of T_0 and T_1 lines. **a** *hpt*II specific PCR showing ~600 bp amplified products; and **b** *WsSQS* specific PCR showing ~1.6 kb amplified products. M: Low range molecular weight ladder (Banglore Genei, India); P: Positive control (plasmid pCAMBIA 1301 carrying *WsSQS* insert); N: Negative control (wild-type plant); 1–10: putative T_0 transformed lines; and 11–13: T_1 individuals

analyses, and the mean values of three control plants were used for each experiments.

Although, regeneration has been reported frequently in *W. somnifera* (Kanungo and Sahoo 2011; Sivanesan 2007), transformation studies were not carried out in any one of them. Genetic transformation of *W. somnifera* for increased production of withanolides has been confined to hairy roots through *A. rhizogenes* (Bandyopadhyay et al. 2007; Murthy et al. 2008), however, hairy root cultures normally produce chemicals which are synthesized in the roots of intact plants. None of previous reports on transformation in *W. somnifera* resulted in stable and successful regeneration of plants from the transgenic tissue. There are only two reports on *A. tumefaciens*-mediated transformation in *W. somnifera* where in one study, the wild type strain of *A. tumefaciens* resulted in the formation of shooty teratomas (Ray and Jha 1999) while another report demonstrated very low transformation efficiency using leaf explants from green house grown plants (Pandey et al. 2010). Obtaining good transformation efficiency has always been a major concern and prerequisite for manipulating the SM biosynthetic pathways. The higher transformation efficiency in the present study can be attributed to the efficient axillary proliferation method employed which utilized apical and nodal segments derived from in vitro raised *Withania* plants. Rapid proliferation system provides large number of genetically identical explants for transformation. Explant selection is another factor that is important for successful transformation and plant regeneration. The present study used apical and nodal segments as explants. The use of organized meristematic tissues maintains cultivar integrity, and has the benefit of rapid shoot elongation and proliferation, thus reducing the chances of somaclonal variations (Sivanesan 2007). Apical and nodal segments are considered to be the ideal for achieving higher transformation efficiency with the proper concentrations of growth regulators (Patel et al. 2014). Nodal explants have also shown to form multiple shoots both from pre-existing and *de novo* buds on MS medium containing 0.1–0.5 mg l^{-1} BAP (Kulkarni et al. 2000). The greatest number of shoots were obtained from the nodal explants in MS medium supplemented with phytohormones (Kumar et al. 2011; Manickam et al. 2000; Sivanesan and Murugesan 2008). The age and type of donor tissue also play crucial role in biochemistry of subsequent cultures lines. *In vitro* cultures have the differential capacity to synthesize SMs depending on the morphological nature of type of explant utilized. The plantlets regenerated from apical and nodal explants have been shown to possess higher content of Withanolide A and Withanone (Sharada et al. 2007).

Accumulation of WsSQS mRNA in transformed tissues

The accumulation of *WsSQS* mRNAs in control and *WsSQS* transformed lines was analyzed by qRT-PCR. In control plants, the maximum expression of *WsSQS* was observed in root followed by leaf and stem (Fig. 4). Root and leaf tissues accumulated 4 and 2 fold higher *WsSQS* mRNA than stem, respectively. Of all the *WsSQS* transformed T_0 plants analyzed by qRT-PCR, three best performing lines ($T_0$20, $T_0$58 and $T_0$79) are shown where the mRNA levels increased up to 2–5 fold (Fig. 4). Similar results were obtained with *Eleutherococcus senticosus* (Seo et al. 2005) and *Panax ginseng* (Lee et al. 2004), where transformation with *SQS*, resulted in accumulation of *SQS* mRNA in the leaves and adventitious roots of the transgenic plants, respectively. This was later observed in *Bupleurum falcatum* also, where *SQS* mRNA expression was enhanced in sense transgenic roots but suppressed in antisense roots as compared to non-transgenic roots (Kim et al. 2011). In the present study, almost 3.5 and 2.5 fold

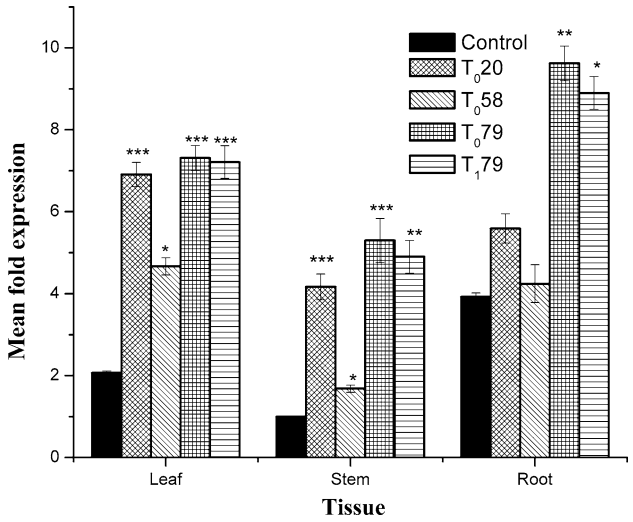

Fig. 4 Tissue specific *WsSQS* transcript analysis in transformed *W. somnifera* T_0 and T_1 lines by qRT-PCR. Ubiquitin gene was used as an internal control. Three best performing T_0 transformed line ($T_0$20, $T_0$58 and $T_0$79) and one T_1 progeny ($T_1$79) were chosen to study mRNA accumulation against the control plant. Values are the means of three replicate measurements and *error bars* show the SE of the mean. *Asterisk* indicates significant differences, (*) for $P < 0.05$; (**) for $P < 0.001$; (***) for $P < 0.0001$, based on Student's *t* test

increase in *WsSQS* mRNA was observed in leaf and root tissue of *WsSQS* transformed lines, respectively. Although, stem showed the maximum fold increase (about 5 fold), the maximum expression of *WsSQS* was observed in root than rest of the tissues (Fig. 4). These results demonstrate that *WsSQS*, catalyzing the regulatory step leading to withanolide biosynthesis, was up-regulated in all the transformed tissues.

WsSQS protein levels and enzyme activity in transformed plants

Indirect ELISA was performed to investigate the relative increase of WsSQS protein levels in *WsSQS* overexpressing T_0 transformed tissues. The antisera dilution 1:5000 was found to contain optimum antibody titer for carrying out the immunological studies. The control tissues showed almost equal expression of WsSQS in root and leaf, which was 2 fold greater than stem. It was found different from the transcript expression pattern in control tissues where root showed highest mRNA levels followed by leaf and stem. There was no direct co-relation found between mRNA expression and protein accumulation patterns, however, consistent with the transcript expression results, ELISA showed increased protein levels in all the *WsSQS* overexpressing transformed tissues, with maximum accumulation in leaf followed by root and stem. Absolute quantification of protein was carried out by plotting the standard curve. In *WsSQS* transformed leaf, WsSQS increased from 1.3 to 3.6 μg g^{-1} fr wt, in stem from 0.51 to 1.2 μg g^{-1} fr wt, and in root from 1.42 to 3.6 μg g^{-1} fr wt (Fig. 5a). This overexpression was further witnessed by western blotting (Fig. 5b). The recombinant truncated WsSQS protein (44 kDa) was used as size marker. Different tissues of $T_0$79 showed clear immune-precipitated protein bands with the higher intensity corresponding to the expected size (~47 kDa).

SQS activity was measured flourimetrically by calculating the amount of NADPH consumed in the reaction, as well as detecting squalene formation in these reactions by GC–MS (see Supplementary Fig. 1). The maximum increase in enzyme activity was found to be 3.3 fold in root tissue followed by 2.7 fold in leaf and 2.1 fold in stem

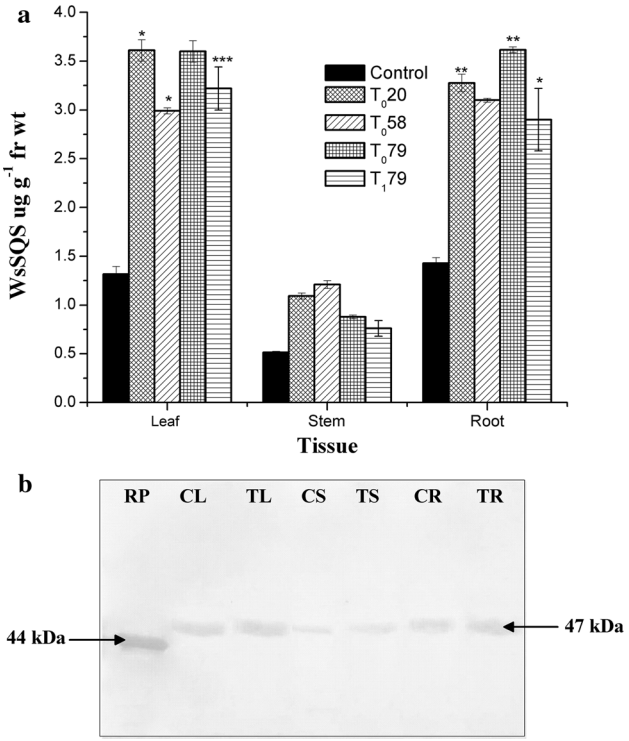

Fig. 5 WsSQS protein expression analyses. **a** WsSQS protein quantification in total soluble protein extracted from different tissues of control and *WsSQS* transformed lines by ELISA, determined from the standard curve plotted between purified recombinant WsSQS protein concentration and absorbance at 405 nm. Values are the means of three replicates from each biological sample and error bars show the SE of the mean. *Asterisk* indicates significant differences, (*) for $P < 0.05$; (**) for $P < 0.001$; (***) for $P < 0.0001$, based on Student's *t* test; and **b** Western blot analysis of *W. somnifera* transformed with pCAMBIA 1301 harboring *WsSQS* gene. Total soluble protein (50 μg) from different tissues of $T_0$79 and control plant was immunodetected with antisera against recombinant WsSQS (1:5000 dilution). *RP* recombinant truncated WsSQS protein as size marker, *CL* control leaf, *TL WsSQS* transformed leaf, *CS* control stem, *TS WsSQS* transformed stem, *CR* control root; and *TR WsSQS* transformed root

(Table 1). The results obtained clearly indicated the increased SQS content in the T_0 *WsSQS* transformed lines (Table 1), which is a consequence of the increased expression of *WsSQS*. Likewise, the enzyme activity was 1.5–3 fold higher and 1.2–3 fold higher in transgenic embryos and transgenic plants of *E. senticosus*, respectively (Seo et al. 2005). Similar results were obtained with *P. ginseng* (Lee et al. 2004) and *B. falcatum* (Kim et al. 2011).

Tissue specific accumulation of withanolides in transformed *W. somnifera*

The present study offers a simple analytical LC–MS system providing well-resolved and symmetrical peaks required for accurate quantitative analysis of withanolides. This allowed identification of four withanolides in the plant extract which were confirmed by retention time (RT) and mass spectrum of their respective standards facilitating their quantification: Withaferin A (11.31 min), Withanolide A (12.35 min), Withanolide B (15.03 min) and Withanone (18.35 min) (see Supplementary Fig. 2). The metabolite profiles indicated variability between the different tissues but their level was found to be higher than control (see Supplementary Fig. 2). The overexpression of *WsSQS* resulted in up to 2 fold increase in total withanolide content of *WsSQS* T_0 transformed plants. The total withanolide content of control leaf, stem and root was 1.84, 2.24 and 2.25 mg g^{-1} dry wt of the tissue, respectively, which increased to 3.55, 3.37 and 3.98 mg g^{-1} dry wt of their respective tissue of $T_0$79 (Fig. 6). The total withanolide increase of about 1.9 fold was achieved in leaf tissue followed by 1.8 in root and 1.5 in stem. The leaf tissue showed maximum fold increase but the overall concentration of total withanolides was highest in *WsSQS* transformed root (Fig. 6). The major change in concentration of withanolides was found in leaf where Withaferin A and Withanolide A increased up to 4–4.5 folds while there was negligible change in Withanolide B and Withanone. This pattern also reflected in root where Withaferin A and Withanolide A increased up to 2–2.5 fold whereas the concentration of Withanolide B and Withanone reached only 1.2 and 1.3 fold, respectively. The metabolite profile of stem was completely different from leaf and root where although Withaferin A and Withanolide B increased to about 2 and 1.5 fold, respectively, the quantity of Withanolide A was found to be almost same, while the concentration of Withanone increased to 3.7 fold. There are previous reports on tissue specific accumulation of SMs in *W. somnifera* where in one study, root tissue accumulated maximum Withanolide A and Withaferin A (Manwar et al. 2012), which is in sharp contrast to another study where the plant was found to accumulate these withanolides more in leaf tissue (Sharma et al. 2007).

Analysis of *W. somnifera* T_1 plants

The primary transformants showing increased withanolides content were raised to advance to the T_1 progeny by selfing the seeds under green house conditions. DNA was extracted from T_1 progeny plants, and subjected to *hpt*II and *WsSQS* specific PCR, as described in Materials and Methods section, which showed an amplification of ~600 bp (Fig. 3a) and ~1.6 Kb (Fig. 3b), respectively; thus confirming the inheritance of inserted genes in the progeny. It was further verified by monitoring T_1 individuals for the increased expression of mRNA and protein, and withanolides production. The data of $T_1$79 is presented here which is the progeny of maximum withanolides producing T_0 plant. qRT-PCR of T_1 progeny showed *WsSQS* mRNA expression levels similar to T_0 lines, suggesting a stable expression of gene in T_1 line (Fig. 4). ELISA analysis of $T_1$79 indicated that the protein expression remained at par with that of T_0 plants. The protein quantity ranged from 0.75 to 3.25 μg g^{-1} fr wt of the tissue (Fig. 5a). The slight variation in the expression is acceptable which may be arising due to the developmental stage of the plant and

Table 1 Summary of WsSQS activity and squalene formed in the enzyme reactions catalyzed by WsSQS isolated from different tissues of control and $T_0$79 plants

Sample	WsSQS activity (pKat mg^{-1} protein)	WsSQS activity (pKat g^{-1} fr wt)	Squalene (μmol mg^{-1} protein)
Control leaf	36 ± 2.1[a]	0.72 ± 0.09[a]	54 ± 3.4
Transformed leaf	54.7 ± 2.5[a]	1.98 ± 0.12[a]	82 ± 6.7
Control stem	10 ± 0.41	0.39 ± 0.01	15 ± 8.9
Transformed stem	14.4 ± 0.86	0.83 ± 0.02	23 ± 1.2
Control root	41 ± 1.5[b]	0.82 ± 0.02[b]	29 ± 1.2[c]
Transformed root	139.4 ± 10.6[b]	2.78 ± 0.32[b]	97 ± 3.4[c]

The control is a tissue transformed with *Agrobacterium* harboring the empty vector. The enzymes activity was calculated in pKat mg^{-1} protein which was validated by quantifying squalene (μmol mg^{-1} protein) formed in each reaction. The fold expression of WsSQS g^{-1} fr wt of tissue was compared. SE of three independent experiments are shown. Values with different letters are significantly different (Student's *t* test, $P < 0.05$)

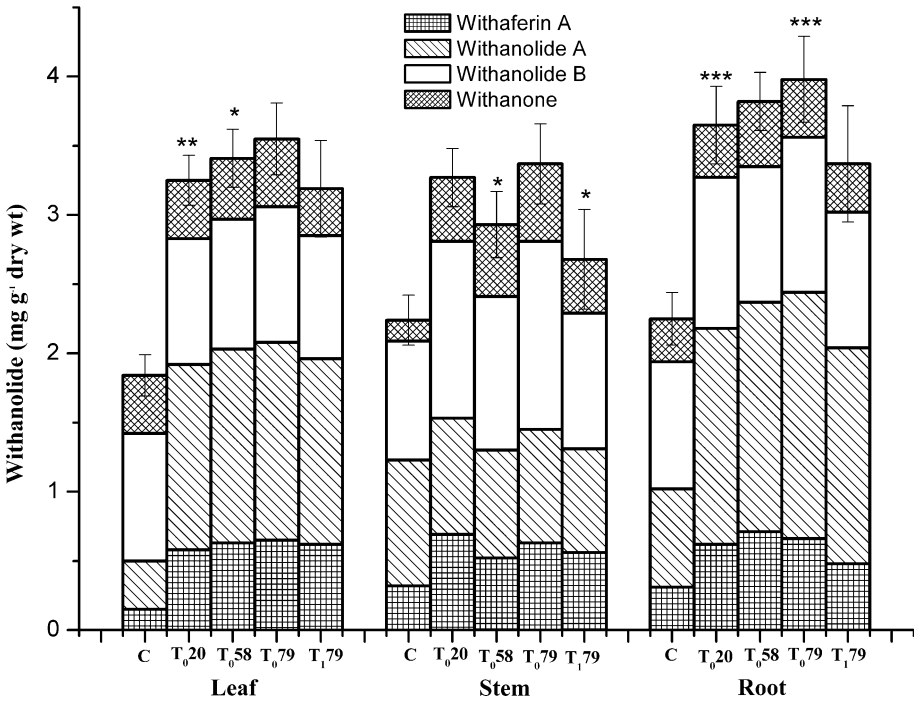

Fig. 6 Improved production of withanolides in T_0 and T_1 transformed tissues overexpressing *WsSQS*. Four different withanolides are shown in leaf, stem and root tissue of control (C), three transformed lines ($T_0$20, $T_0$58 and $T_0$79) and one T_1 progeny ($T_1$79) where $T_0$79 is the best performing line. The control line (C) is a tissue transformed with *Agrobacterium* harboring the empty vector. *Vertical bars* indicate the mean values ± SE from three independent experiments. *Asterisk* indicates significant differences, (*) for $P < 0.05$; (**) for $P < 0.001$; (***) for $P < 0.0001$, based on Student's *t* test

season of growth. LC–MS of T_1 plants was conducted to identify the plants that had retained increased withanolide content which was found more or less consistent with T_0 plants (Fig. 6).

There are few reports on plant cell and hairy root cultures initiated for the increased production of SM in *Withania*. Murthy et al. (2008) developed transformed hairy root cultures of *W. somnifera* which produced 2.7 fold higher Withanolide A. In another study, *W. somnifera* transformation with *A. tumefaciens* resulted in formation of shooty teratomas which accumulated Withaferin A and Withanolide D, and rooty teratomas with Withanolide D, but the levels of these withanolides was low (Ray and Jha 1999). The overexpression of *Arabidopsis thaliana* SS1 gene (encoding SQS) in *W. coagulans* using *A. rhizogenes* resulted in increased phytosterol and withanolide production in engineered hairy roots, thus indicating the potentiality of SQS to promote triterpenoid biosynthesis (Mirjalili et al. 2011). None of the previous studies on hairy root and plant cell cultures have resulted in significant levels of the metabolites required for their economic exploitation. Moreover, an important constraint in the commercial utilization of hairy root culture is development and up-scaling of appropriate vessels for the delicate and sensitive hairy roots.

Metabolic engineering of withanolide biosynthetic pathway is an alternative approach for their improvement. SQS operates at a branch point of the pathway regulating the metabolic flux and catalyzes the first committed step leading to the synthesis of different withanolides (Mirjalili et al. 2009). Thus, manipulating the expression of *SQS* could be a tool for modifying the SMs of this plant. This paper describes the metabolic engineering of isoprenoid pathway by overexpressing *SQS* which resulted in enhanced production of withanolides in *W. somnifera*. Similar results were obtained with *P. ginseng* (Lee et al. 2004) and *E. senticosus* (Seo et al. 2005) where the transformed plants accumulated 1.6–3 fold higher total ginsenoside content in adventitious roots and 2–2.5 times higher triterpene saponin, respectively. Likewise, transgenic roots of *B. falcatum* overexpressing *BfSS1* in the sense orientation resulted in *SQS* mRNA accumulation and enhanced production of both phytosterol and saikosaponins (Kim et al. 2011). Overexpression of *SQS* in *Glycyrrhiza uralensis* led to 2.6 fold higher glycyrrhizin content as compared to control hairy roots (Lu et al. 2008). There is only one report of *SQS* overexpression in *W. somnifera* which resulted in 2.5 fold increase in Withanolide A and production of Withaferin A from the transformed callus and cell suspension cultures (Grover et al. 2013). Cell suspension cultures are more prone to contamination and are difficult to maintain, and callus is a chimeric tissue, thus reducing the complete effect of total number of transformed cells actually contributing in improved withanolide production. The fate of developing cells in callus is unknown, thus providing no idea about the specific tissue involved in the production of SMs. It has also been reported previously that transformed undifferentiated callus and cell suspension cultures of *W. somnifera* are generally unable to synthesize withanolides (Bandyopadhyay et al.

2007; Roja et al. 1991). The major drawback of callus culture is their tendency to undergo frequent genetic erosions (D'Amato 1977), and therefore, pure clones cannot be maintained. So callus culture could be limited source of metabolites while transformed plant production is suitable option for conservation of germplasm. Development of whole transformed plants presents a one step ahead and convenient technology for steady supply of bioactive compounds.

In summary, the present study establishes significant involvement of SQS in withanolide biosynthesis. Previously, we reported the isolation and cloning of *WsSQS* from *W. somnifera* (Gupta et al. 2012), and here we demonstrate enhanced withanolides in *W. somnifera* by overexpressing squalene synthase. Such plant metabolic engineering studies can be used as a tool to improve the production of *Withania* bioactive compounds, as well as obtain greater understanding of secondary metabolism, in general.

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Conflict of interest The authors declare that they have no conflict of interest and the publication of the work has been approved by all co-authors.

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